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<b>(54) Title:</b> ISOLATION OF MAMMALIAN HEMATOPOIETIC STEM CELLS			
<b>(57) Abstract</b> <p>The present invention relates to a method of purifying mammalian hematopoietic stem cells from a bone marrow cell population. The present invention also relates to a method of separating cycling HSCs from quiescent HSCs in a purified mammalian HSC sample. Further, the invention relates to a method of identifying an agent which is a fluorescent, vital dye which is lipophilic for use in purifying mammalian HSCs. The present invention also relates to a method of transplanting bone marrow in a mammalian host, such as a human, comprising introducing into the host the purified HSCs described herein. The invention further relates to a method of <i>in vivo</i> administration of a protein comprising transfecting a purified HSC with a construct comprising DNA which encodes a protein of interest and then introducing the stem cell into the host where the protein of interest is expressed. The present invention also relates to hematopoietic stem cells purified using the methods described herein.</p>			

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## ISOLATION OF MAMMALIAN HEMATOPOIETIC STEM CELLS

RELATED APPLICATIONS

This application is a Continuation-in-Part of  
copen ding U.S. Patent Application Serial No. 08/471,758,  
5 filed June 6, 1995, the entire teachings of which are  
incorporated herein by reference.

BACKGROUND OF THE INVENTION

Hematopoietic stem cells (HSC) are multipotential  
cells which reside in the bone marrow and replenish all  
10 adult hematopoietic lineages through the lifetime of an  
animal. Lack of a purified population of stem cells has  
hampered definitive understanding of the factors which  
regulate their growth and differentiation. Several schemes  
for the enrichment of hematopoietic stem cells from murine  
15 bone marrow have been developed, but a phenotypically  
homogeneous population of stem cells has not been  
identified to date (Visser, J.W., et al., *J. Exp. Med.*,  
159:1576-90 (1984); Pallavicini, M.G., et al., *Exp.*  
*Hematol.*, 13:1173-81 (1985); Spangrude, G.J., et al.,  
20 *Science*, 241:58-62 (1988); Szilvassy, S.J., et al., *Blood*,  
74:930-9 (1989); Jones, R.J., et al., *Nature*, 347:188-9  
(1990); Nicola, N.A., et al., *Blood*, 58:376-86 (1981);  
Bertoncello, L., et al., *Exp. Hematol.*, 13:999-1006  
(1985)).

25 The ability to obtain a phenotypically homogeneous  
population of stem cells is important in achieving greater  
success with bone marrow transplants (BMTs). For example,  
autologous BMT could become a candidate for use in leukemic  
and other cancer therapies if it were possible to  
30 definitively remove cancerous cells from the bone marrow.

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Isolation of pure reconstituting HSCs may allow this. Furthermore, non-autologous BMT may also benefit from HSC purification, as transplantation of purified HSCs may ameliorate graft versus host disease (GVH) syndromes. In addition, a homogeneous stem cell population would be advantageous for hematopoietic stem cell gene therapy. Previous attempts to infect unmanipulated murine and human HSCs with recombinant retroviruses have met with mixed success, presumably at least in part due to the inability of retroviral vectors to integrate into quiescent HSCs, which are widely held to constitute the majority of the HSC population. Thus, purification of HSCs and the ability to distinguish between quiescent HSCs and cycling HSCs would have important implications for HSC gene therapy.

#### 15 SUMMARY OF THE INVENTION

The present invention is based on the discovery that unusual uptake and fluorescence properties of a dye in hematopoietic stem cells (HSCs) make it possible to purify HSCs and isolate a subpopulation of HSCs that are naturally replicating *in vivo*. Thus, the invention relates to a method of purifying mammalian hematopoietic stem cells from a bone marrow cell population.

In one embodiment, a bone marrow cell sample is obtained and combined with a fluorescent, lipophilic vital dye which is a substrate for a multiple drug resistant protein (i.e., mdr protein) under conditions appropriate for cells to take up the dye. The term "substrate" is defined herein as a substance which is removed by mdr. The resulting combination is exposed to an excitation wavelength which results in fluorescence of the dye, which is measured using an emission wavelength. The amount of dye contained by each population of cells resolved with the emission wavelength is determined. The population of

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nucleated cells which contains the smallest amount of dye at the emission wavelength is purified, mammalian HSCs.

- In another embodiment a bone marrow cell sample is obtained and combined with a fluorescent, lipophilic vital dye which is a substrate for a mdr protein, under conditions appropriate for cells to take up the dye. The resulting combination is exposed to an excitation wavelength which results in fluorescence of the dye, which is measured using two emission wavelengths simultaneously.
- 10 The amount of dye contained by each population of cells resolved with the two emission wavelengths is determined. The population of nucleated cells which contains the smallest amount of dye at both wavelengths is purified, mammalian HSCs.
- 15 The present invention also relates to a method of separating cycling HSCs from quiescent HSCs in a purified mammalian HSC sample. In the method, a purified HSC sample is obtained and combined with a fluorescent, lipophilic vital dye which binds DNA and is a substrate for a mdr
- 20 protein and an inhibitor of the mdr protein under conditions appropriate for the cells to take up the dye and the inhibitor. The amount of dye present in the purified HSCs is measured, wherein purified HSCs which contain the smallest amount of dye have a  $2n$  quantity of DNA (i.e.,
- 25 which refers to the base number of chromosomes in a cell) and are designated as quiescent HSCs, and purified HSCs which contain a greater amount of dye have a greater amount of DNA (i.e.  $>2n$ ) and are designated cycling HSCs. This would also apply to obtaining cycling cells from a
- 30 population of cells having high mdr activity. For example, a heterogeneous population of cells such as bone marrow can be combined with the dye and the inhibitor. Cycling and non-cycling populations are determined as described above. In this case a heterogenous population of cycling cells are
- 35 isolated and can be used for infecting with retroviruses.

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Further, the invention relates to a method of identifying an agent which is a fluorescent, vital dye which is lipophilic for use in purifying mammalian HSCs. In one embodiment, a bone marrow cell sample is obtained and combined with the agent under conditions appropriate for the cells to take up the agent. The resulting combination is exposed to an excitation wavelength which results in fluorescence of the agent, which is measured using an emission wavelength and the amount of dye exhibited by each population of cells is determined. If distinct populations of nucleated cells are observed in which one of the population of cells contains the smallest amount of dye and the population is purified, mammalian HSCs, then the agent is a fluorescent vital dye which is lipophilic for use in purifying HSCs.

In another embodiment, a bone marrow cell sample is obtained and combined with the agent under conditions appropriate for the cells to take up the agent. The resulting combination is exposed to an excitation wavelength which results in fluorescence of the agent which is measured using two emission wavelengths simultaneously. The amount of dye contained by each population of cells resolved with the two emission wavelengths is determined. If distinct populations of nucleated cells are observed in which one of the population of cells contains the smallest amount of dye and the population is purified, mammalian HSCs, then the agent is a fluorescent, vital dye which is lipophilic for use in purifying HSCs.

The present invention also relates to a method of transplanting bone marrow in a mammalian host, such as a human, comprising introducing into the host the purified HSCs described herein. The invention further relates to a method of in vivo administration of a protein comprising infecting or transfecting a purified HSC with a construct comprising DNA or RNA which encodes a protein of interest

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and introducing the infected or transfected purified HSC into the host, in which the protein of interest is expressed. In a particular embodiment, exogenous DNA encoding a protein of interest is infected or transfected  
5 into a cycling HSC.

The present invention also relates to hematopoietic stem cells purified using or obtainable by (obtained by) the methods described herein. In one embodiment, the purified HSCs of the present invention are CD34<sup>neg</sup>. In  
10 another embodiment, the purified HSCs are side population (SP) cells. In another embodiment, the purified HSCs have at least one of the following characteristics: glycophorin A<sup>neg</sup>, CD38<sup>low/neg</sup>, CD13<sup>neg</sup>, CD15<sup>neg</sup>, CD19<sup>neg</sup> and CD20<sup>neg</sup>.

Thus, the present invention allows for the use of  
15 purified HSCs in bone marrow transplants. In addition, the method of separating cycling HSCs from quiescent HSCs provides a means of obtaining cycling HSCs, separated from quiescent HSCs for transfection with a construct encoding a protein of interest. As discussed above, recombinant  
20 retroviral are unable to integrate into quiescent HSCs. Thus, the ability to specifically transfect cycling HSCs will provide greater success in in vivo administration of proteins to or by HSCs.

#### BRIEF DESCRIPTION OF THE FIGURES

25 Figure 1A is a graph of Hoechst red versus blue fluorescence on a linear scale of whole murine normal bone marrow stained with Hoechst 33342 stain, in which the boxed region represents the hematopoietic stem cell activity, which is 0.1% of the total bone marrow cell population.

30 Figure 1B is a graph illustrating the analysis of Sca-1 and lineage marker staining of whole murine normal bone marrow.

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Figure 1C is a graph of Hoechst red versus blue fluorescence on a linear scale of whole murine normal bone marrow stained with Hoechst 33342 stain, in which the region indicated in Figure 1B is used as a live gate in conjunction with the gate to exclude red and dead cells.

Figure 1D is a graph illustrating the analysis of Sca-1 and lineage marker staining of whole murine normal bone marrow cells which fall exclusively into the boxed region indicated in Figure 1A.

Figure 1E is a graph of Hoechst red versus blue fluorescence on a linear scale of Sca-1 enriched murine normal bone marrow stained with Hoechst 33342 stain, in which the specific side region indicated in Table 2 is delineated.

Figure 2A is a graph of Hoechst red versus blue fluorescence on a linear scale of whole bone marrow stained with Hoechst 33342.

Figure 2B is a graph of Hoechst red versus blue fluorescence on a linear scale of whole bone marrow stained with Hoechst 33342 in the presence of Verapamil.

Figure 3A is a graph of Hoechst red versus blue fluorescence on a linear scale of purified HSCs restained with Hoechst 33342 in the presence of verapamil.

Figure 3B is a graph of propidium iodide (PI) versus cell number of purified HSC stained with PI.

Figure 4A is a graph of Hoechst red versus blue fluorescence on a linear scale of human whole bone marrow cells stained with Hoescht 33342 stain.

Figure 4B is a graph of Hoechst red versus blue fluorescence on a linear scale of human cord blood cells stained with Hoescht 33342 stain.

Figure 5A is a graph of Hoechst red versus blue fluorescence on a linear scale of porcine bone marrow cells stained with Hoescht 33342 stain.



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Figure 5B is a graph of Hoechst red versus blue fluorescence on a linear scale of Rhesus bone marrow cells stained with Hoescht 33342 stain.

DETAILED DESCRIPTION OF THE INVENTION

5       The invention relates to a method of purifying mammalian hematopoietic stem cells (HSCs) from a bone marrow cell population.

10       In one embodiment, a bone marrow cell sample is obtained and combined with a fluorescent, lipophilic vital dye which is a substrate for a multiple drug resistant protein (i.e., mdr protein) under conditions appropriate for cells to take up the dye. The term "substrate" is defined herein as a substance which is removed from a cell by mdr. The resulting combination is exposed to an  
15       excitation wavelength which results in fluorescence of the dye, which is measured at an emission wavelength and the amount of dye exhibited by each population of cells is determined. The population of nucleated cells which contains the smallest amount of dye at the emission  
20       wavelength is purified, mammalian HSCs.

25       In another embodiment, a bone marrow cell sample is obtained and combined with a fluorescent, lipophilic vital dye which is a substrate for a mdr protein, under conditions appropriate for cells to take up the dye. The  
30       resulting combination is exposed to an excitation wavelength which results in fluorescence of the dye, which is measured using two emission wavelengths simultaneously. The amount of dye contained by each population of cells resolved with the two emission wavelengths is determined. The population of nucleated cells which contains the  
smallest amount of dye at both wavelengths is purified, mammalian HSCs. As described herein, the method of purifying HSCs has been used to purify side population (SP) cells from four types of mammals: murine, human, monkey and

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porcine. Characterization of the murine SP cells indicate they are purified HSCs. Further characterization of the human, monkey and porcine SP cells will confirm they are purified HSCs. Applicants have shown that the purified

5 HSCs described herein successfully repopulated stem cell activity in lethally irradiated recipients, providing 1000 fold enrichment of HSC activity in mouse bone marrow. In addition, the HSCs of the present invention have been shown to allow survival of lethally irradiated recipients.

10 Thus, the present invention relates to a method for the isolation or purification of mammalian HSCs, and is based on the use of FACS analysis of bone marrow cells stained with a fluorescent vital dye. Starting with untreated bone marrow, 1,000 fold enrichment of *in vivo* HSC

15 reconstitution activity can be consistently achieved. The purification strategy has also led to a method for separating cycling from quiescent stem cells, which will facilitate efficient gene transfer into hematopoietic stem cells.

20 The dye which can be used in the method for purifying HSCs is a fluorescent, vital dye which is a substrate for a *mdr* protein. Preferably, the dye is also lipophilic. In the method of separating cycling HSCs from quiescent HSCs, the dye must also be a DNA binding dye. In some instances,

25 one dye can be used in the method for purifying HSCs and in the method for separating cycling HSCs from quiescent HSCs. For example, as described in Examples 1 and 3, Hoechst 33342 dye (Hoechst dye) can be used in both the method for purifying HSCs and the method of separating cycling HSCs

30 from quiescent HSCs. Although the method of the present invention is exemplified using Hoechst dye (Example 1), other dyes can be used to practice the methods of the present invention. For example, Rhodamine 123 might be used to practice the invention, particularly in the

35 embodiment in which purified HSCs are isolated.

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Hoechst 33342 is a fluorescent DNA-binding dye useful for flow cytometry analysis. The dye is readily taken up by live cells, in which it binds to DNA. The quantity of Hoechst 33342 fluorescence relates to DNA content in a cell and, therefore, is an indicator of cell cycle. However, as described in Example 1, when Hoechst dye was used to examine DNA content in murine bone marrow cells by standard methods, a complex fluorescence pattern was observed. When dye fluorescence was observed simultaneously at two emission wavelengths (the red and the blue), several distinct populations could be resolved. These populations are shifted relative to each other predominately with regard to fluorescence on the Hoechst "red" axis (Fig. 1A). Analysis of hematopoietic cells in this staining profile indicated that one population of cells, boxed in Figure 1A, was predominately Sca-1+lin<sup>low</sup> (Fig. 1D). That is, 75% of the cells from the boxed region in Fig. 1A expressed Ly-6A.2 (Sca-1+), and a low level of six lineage antigens (detected by a cocktail of antibodies against B220, Gr-1, Mac-1, CD4, CD5, and CD8). These cell surface characteristics have previously been identified on murine hematopoietic stem cells by Applicants and others (Spangrude, G.J., et al., *Science*, 241:58-62 (1988); Uchida, N. and Weissman, I.L., *J. Exp. Med.*, 175:175-84 (1992)). Characterization of the purified human SP described herein indicate that the purified HSCs are: glycophorin A<sup>neg</sup>, CD38<sup>low/neg</sup>, CD13<sup>neg</sup>, CD15<sup>neg</sup>, CD19<sup>neg</sup>, CD20<sup>neg</sup>, and CD34<sup>neg</sup>. Characterization of purified HSCs in mouse indicate that the purified mouse HSCs are: c-kit<sup>pos</sup>, Sca-1<sup>pos</sup>, Gr-1<sup>neg/low</sup>, Mac-1<sup>neg/low</sup>, CD4<sup>neg/low</sup>, CD8<sup>neg</sup>, B220<sup>neg</sup>, CD5<sup>neg</sup>, CD43<sup>pos</sup>, CD45<sup>pos</sup>, M169<sup>pos</sup>, AA4<sup>neg/low</sup>, class I MHC<sup>pos</sup>, class II MHC<sup>neg</sup>, Rhodamine 123<sup>low</sup>, and Wheat germ agglutinin (WGA)<sup>pos</sup>. This population, referred to as the side population, or SP, represents 0.1% of total bone marrow. These SP cells were

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also a sub-population (approximately 5%) of the total Sca-1<sup>+</sup>, lin<sup>low</sup> cells in the bone marrow.

To examine the functional properties of these cells, long term bone marrow transplantation experiments were performed, as described in Example 2. A competitive repopulation assay, in which defined numbers of purified cells (i.e., SP cells) were transplanted into lethally irradiated recipients along with unfractionated but distinguishable whole bone marrow cells (i.e., unfractionated cells), was used. In this semi-quantitative method, the relative stem cell activity of sorted or SP cells versus unfractionated cells is assessed by determining the percentage of peripheral blood cells descended from each of the two input populations in the bone marrow transplant recipients (Harrison, D.E., *Blood*, 55:77-81 (1980); Harrison, D.E., et al., *Proc. Natl. Acad. Sci. U.S.A.*, 85:822-826 (1988)). It is generally accepted that at four months post-transplant, the majority of peripheral blood cells are derived from long term reconstituting stem cells introduced in the transplant instead of from committed progenitors. In addition, differentiated cells and progenitors in the unfractionated competitor bone marrow rescue recipients from the otherwise lethal irradiation. This allows the long term multilineage repopulating functions of the input HSC to be examined separately from short-term activities.

As described in Example 2, in competitive repopulation assays with the SP cells, average enrichments of HSC activity of approximately 1,000 fold over multiple experiments were achieved (Table 1). Since the SP cells represent 0.1% of the total bone marrow, this indicates that most if not all reconstituting cells in normal mouse bone marrow reside in the SP fraction. Moreover, competitive repopulation experiments with other Hoechst-

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stained populations shown in Fig. 1A, revealed almost no additional stem cell activity. See Table 2.

Having established by competitive repopulation that stem cell activity fell into the SP region, the ability of that population of cells to protect recipients from lethal irradiation (i.e., radioprotection) was investigated. As shown by data from three separate experiments in Table 3, approximately 150 purified cells protected 50% of the recipients from lethal irradiation. That is, the presence of approximately 150 purified cells in the lethally irradiated recipients resulted in survival of 50% of the recipients.

It was of interest to account for the unusual Hoechst-staining properties of the hematopoietic stem cells. Since it is known that several vital dyes are actively pumped out of the cells by the *mdr* protein (i.e., *p*-glycoprotein) (Chaudhary, P.M. and Roninson, L.B., *Cell*, 66:85-94 (1991)), the possibility that the exceptionally low Hoechst fluorescence was due to higher *mdr* activity in murine hematopoietic stem cells was investigated. As described in Example 3, bone marrow stained with Hoechst 33342 in the absence or presence of the drug verapamil, an inhibitor of *mdr*, demonstrates that the stem cell population is visible (i.e., able to be isolated) in the absence of verapamil (Fig. 2A, Fig. 2B).

Based upon the ability of verapamil to block the efflux of Hoechst dye, it was postulated that separation of replicating stem cells from quiescent stem cells is possible. As discovered and described herein, this has been shown to be correct, and can further be used to separate replicating stem cells from quiescent stem cells. That is, as shown herein, in the presence of verapamil, the efflux activity of Hoechst dye is blocked, and a higher concentration of Hoechst dye is retained in the cells. In cell populations which do not contain high amounts of *mdr*

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activity, Hoechst dye reflects DNA content and has commonly been used in cell cycle analysis. Cycling cells have a greater amount of DNA than quiescent cells. Thus, a greater amount of dye will be present in cycling HSCs resulting in greater fluorescence of the dye present in the cycling HSCs when the dye is excited at the appropriate wavelength. Conversely, a lower amount of dye will be present in quiescent cells resulting in lower fluorescence of the dye present in quiescent HSCs when the dye is excited at the appropriate wavelength. Thus, measuring the relative amount of fluorescence of a dye in purified HSCs allows for separation of cycling HSCs from quiescent HSCs (Techniques in Cell Cycle Analysis, ed. Gray, J.W. and Darzynkiewicz, Humana Press, Clifton, NJ (1987)).

Accordingly, as described in Example 4, HSCs were purified (sorted) on the basis of low Hoechst staining, to produce SP cells as described herein. The sorted or SP cells were restained with Hoechst dye in the presence of verapamil. Cell-cycle analysis was performed as described in Example 1 and the number of stem cells in S-G<sub>2</sub>M (i.e., cycling HSCs) was found to range between 1 and 3% of the purified cells (Fig. 3A). This figure correlates well with the number shown to be in S-G<sub>2</sub>M by propidium iodide staining of the purified cells (Fig. 3B). Stem cells in G<sub>0</sub>-G<sub>1</sub> (i.e., quiescent HSCs) made up the rest of the purified cells.

With a view to using cycling stem cells as targets for gene transfer, such as retroviral-mediated gene transfer, the relative engraftment potential of stem cells that were in G<sub>0</sub>-G<sub>1</sub> versus S-G<sub>2</sub>M was compared. These subsets of the SP population were purified by flow cytometry using the verapamil-blocking strategy for cell cycle analysis described in Example 1, and tested in the competitive

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repopulation assay described in Example 2. Table 4 shows the results from the experiment.

Thus, the present invention also relates to a method of separating cycling HSCs from quiescent HSCs in a purified stem cell sample. In this method, a purified HSC sample is combined with two substances: a fluorescent, lipophilic vital dye which binds DNA and is a substrate for a mdrl protein and an inhibitor of the mdrl protein. This is carried out under conditions appropriate for the cells to take up the dye and the inhibitor. The amount of dye present in the HSCs is subsequently measured or observed for differences in intensity of fluorescence. The extent to which the dye is contained by a cell is in proportion to the quantity or concentration of DNA in the cell.

Quiescent HSCs contain less or a lower concentration of, DNA than do cycling HSCs and, therefore, quiescent cells take up less dye than is taken up by cycling HSCs. Quiescent HSCs can be distinguished from (i.e., separated from) cycling HSCs on the basis of the quantity or concentration of the vital dye present. Those which contain a lesser quantity or concentration of dye are quiescent HSCs and those which contain a larger quantity or concentration of dye are cycling HSCs. This would also apply to obtaining cycling cells from a population of cells having high mdrl activity. For example, a heterogeneous population of cells such as bone marrow can be combined with the dye and the inhibitor. Cycling and non-cycling populations are determined as described above. In this case a heterogeneous population of cycling cells are isolated and can be used for infecting with retroviruses.

The invention further relates to a method of identifying an agent which is a fluorescent, vital dye for use in purifying hematopoietic stem cells. In one embodiment, a bone marrow cell sample is obtained and combined with the agent under conditions appropriate for

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the cells to take up the agent. The resulting combination is exposed to an excitation wavelength which results in fluorescent of the agent, which is measured using an emission wavelength. The amount of dye contained by each population of cells resolved with the emission wavelength is determined. If distinct populations of nucleated cells are observed in which one of the population of cells contains the smallest amount of dye and the population is purified, mammalian HSCs, then the agent is a fluorescent vital dye which can be used in purifying hematopoietic stem cells.

In another embodiment, an agent is identified by combining a bone marrow cell sample and an agent to be assessed, under conditions appropriate for the cells to take up the agent. The resulting combination is exposed to an excitation wavelength which results in fluorescence of the dye. The dye fluorescence is measured using two emission wavelengths simultaneously, so that distinct populations of live bone marrow cells are resolved on the basis of fluorescence. Dye uptake is assessed in each population of cells. If distinct populations of cells are observed in which one of the population of cells contains the least amount of dye at both wavelengths and the population is purified, mammalian HSCs, then the agent is a fluorescent, vital dye which is for use in purifying HSCs.

The stem cell purification strategy described herein can be used with any suitable mammalian (e.g., vertebrate) species. Applicants have shown that SP cells can be purified from four types of mammals: mice, humans, monkeys and pigs. As described in Example 5, adult human bone marrow cells and human cord blood cells have been stained with Hoechst 33342 and a staining pattern remarkably similar to that which was observed for murine cells was observed for these cells. The frequency of cells in the human side population is also approximately 0.03% (range



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0.01% to 0.05%). As described in Example 6, HSCs from porcine bone marrow and monkey bone marrow were purified by using the method of the present invention. Further characterization of the HSCs purified from monkeys, which is described in Example 7, indicates that greater stem cell activity is in the SP cells which are CD34<sup>pos</sup>.

Obtaining a bone marrow cell sample from a mammal for use in the methods of the present invention can be achieved using routine methods known to those of skill in the art. For example, as described in Example 1, the bone marrow cells can be obtained by extracting a cell suspension from the femurs and/or tibias of the mammal, passing the cells through an orifice (e.g., an 18 gauge needle) and pelleting the cells by centrifugation.

The HSCs for use in the methods of the present invention can be obtained from any suitable mammalian source such as rodent (e.g., rats, mice), primate, dog, pig, cat, monkey, and/or human sources.

In the method of separating cycling and quiescent purified HSCs, the purified HSCs can be obtained using any suitable method for purifying HSCs. For example, the method for purifying HSCs as described herein can be used. Alternatively, a method of purifying HSCs based on cell surface markers can be used (Visser, J.W., et al., *J. Exp. Med.*, 159:1576-90 (1984); Pallavicini, M.G., et al., *Exp. Hematol.*, 13:1173-81 (1985); Spangrude, G.J., et al., *Science*, 241:58-62 (1988); Szilvassy, S.J., et al., *Blood*, 74:930-9 (1989); Jones, R.J., et al., *Nature*, 347:188-9 (1990); Nicola, N.A., et al., *Blood*, 58:376-86 (1981); Bertoncello, L., et al., *Exp. Hematol.*, 13:999-1006 (1985)).

The excitation wavelength used in the method of purifying HSCs, is a suitable wavelength which will excite the particular dye chosen to a measurable extent. For

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example, in the embodiment in which the Hoechst dye is used to purify HSCs, an appropriate excitation wavelength is from about 250 nm to about 450 nm, and in a particular embodiment, is about 350 nm. Hoechst dye emission can be  
5 detected at a range of wavelengths, from about 400 nm to about 700 nm, and in a particular embodiment, about 600 nm. In another embodiment about 450 nm and about 650 nm can be used simultaneously to detect Hoechst dye emission.

In the method of purifying HSCs, the fluorescence of  
10 the dye can be measured at one emission wavelength. Alternatively, two emission wavelengths can be used simultaneously to measure fluorescence of the dye. Suitable emission wavelengths are those which will measure the fluorescence of the dye chosen so that distinct  
15 populations of live bone marrow cells are resolved. For example, in the embodiment in which the Hoechst dye is used to purify HSCs, the fluorescence of the Hoechst dye is measured at two wavelengths using a 450 band pass (BP) 20 and a 675 edge filter long pass (EFLP) optical filter. As indicated by the graph of Figure 1A, the fluorescence of the Hoechst dye can also be measured using only the red  
20 emission wavelength (i.e., 675 nm) to obtain purified HSCs. This was a surprising result since normally 450 nm emission wavelength is used with Hoechst dye because that is its  
25 peak emission wavelength.

The amount of dye used in the methods of the present invention functionally will generally be from about 1  $\mu\text{g/ml}$  to about 20  $\mu\text{g/ml}$  dye, preferably from about 5  $\mu\text{g/ml}$  to about 15  $\mu\text{g/ml}$  dye and in particular, about 5  $\mu\text{g/ml}$  dye.

30 The staining time with the dye (i.e., the length of time cells are exposed to dye) varies depending on the temperature at which staining is to occur and the dye concentration in the methods of the present invention. Thus, staining can occur overnight or over a number of days  
35 at the appropriate temperature. In particular, the

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staining time with the dye can be from about 30 minutes to about 180 minutes, preferably between about 60 minutes to about 120 minutes. In a particular embodiment of the method for purifying HSCs, the staining time with the Hoechst dye is about 90 minutes. In a particular embodiment of the method for separating cycling and quiescent purified HSCs, the staining time with the Hoechst dye is about 60 minutes.

The temperature at which staining with the dye can be carried out is from about 4°C to about 45°C, preferably about 15°C to about 45°C, and in particular, about 37°C in the methods of the present invention. The temperature at which staining with the dye can be carried out can also be room temperature (i.e., about 25°C).

In a particular embodiment of the present method of purifying HSCs, 5 µg Hoechst dye is used to stain a bone marrow cell population and, thus, the HSCs it contains, for 90 minutes at 37°C. In a particular embodiment for the method of separating cycling HSCs from quiescent HSCs, 10 µg Hoechst dye is used to stain the murine bone marrow cell population for 60 minutes at 37°C. In another embodiment, human and monkey bone marrow were stained for 120 minutes at 37°C. In a further embodiment, porcine bone marrow was stained for 90 minutes at 37°C.

In the method of separating cycling HSCs from quiescent HSCs, an inhibitor of the mdr protein is a substance or agent which interferes with the activity of the mdr protein in the HSCs. That is, an inhibitor of the mdr protein is a substance or agent which interferes with the ability of the mdr protein to remove the dye from the HSCs. Inhibitors of the mdr protein include verapamil, antibodies directed against mdr (i.e., anti-multiple drug resistant protein antibody), reserpine, PAK-104P, vincristine and SDZ PSC 833. The term "multiple drug resistant protein" as used herein includes the multiple

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drug resistant (mdr) protein and proteins which exhibit mdr-like activity (i.e., an mdr-like efflux of a dye from a HSC). For example, analogs or derivatives of the mdr protein, are included in the term "multiple drug resistant protein".

In the method of separating cycling HSCs from quiescent HSCs, a low amount or quantity of DNA (i.e.,  $2n$  DNA or base number of chromosomes in a cell) in HSCs indicates the presence of quiescent HSCs (i.e., HSCs cells in the  $G_0$ - $G_1$  phase) and a high amount or quantity of DNA (i.e.,  $>2n$  DNA) in HSCs indicates the presence of cycling HSCs (i.e., HSCs cell in the  $S$ - $G_2M$  phase).

The HSCs obtained by the method of the present invention can be used in a variety of ways. For example, the HSCs of the present invention can be transplanted into a host (e.g., mammal, particularly human) in need of a bone marrow transplant (e.g., an irradiated host or a host undergoing chemotherapy). In addition, the HSCs of the present invention can be used to treat diseases or conditions in which an individual needs bone marrow cells. The present invention further relates to a method of providing a host with purified HSCs comprising the step of introducing into the host the purified HSCs described herein. In particular embodiments, cycling HSCs or quiescent HSCs are introduced into the host.

The methods of the present invention can be used for in vivo administration of protein by transfecting or infecting purified HSCs with recombinant vectors or constructs comprising DNA which encodes a protein of interest. In particular embodiment, cycling or quiescent purified HSCs are transfected or infected with recombinant constructs comprising DNA or RNA which encodes a protein of interest. Previous attempts to infect unmanipulated HSCs with recombinant retroviral vectors have met with mixed

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success, presumably at least in part due to the inability of vectors to integrate into quiescent HSCs, which as shown herein constitute the majority of the HSCs population. Thus, the method of separating cycling HSCs from quiescent HSCs allows for greater success in infecting HSCs with recombinant retroviral vectors. The "administration of protein" by definition includes the delivery of a recombinant HSC which expresses a protein in vivo. For example, a purified cycling HSC containing a vector, wherein the vector contains a DNA or RNA sequence which expresses a protein of interest can be administered to a host under conditions in which the protein of interest is expressed *in vivo* (see e.g., United States patent Number 5,399,346 which is herein incorporated by reference).

Weissman, et al. have worked with Sca-1<sup>positive</sup>, lin<sup>negative</sup>, and Thy-1<sup>low</sup> cells, to characterize murine hematopoietic stem cells. However, it has become increasingly clear that this population is not homogeneous for stem cell activity (Li, C.L. and Johnson, G.R., "Rhodamine 123 reveals heterogeneity within murine Lin<sup>-</sup>, Sca-1<sup>+</sup> hemopoietic stem cells", *J. Exp. Med.* 175:1443-1447 (1992); Spangrude, G.J. and Johnson, G.R., "Resting and activated subsets of mouse multipotent hematopoietic stem cells", *Proc. Natl. Acad Sci. USA* 87:7433-7437 (1990)). These cells can be subfractionated with the use of the vital dye Rhodamine-123, where the 5-10% of cells that contain the lowest amount of the dye after staining have most if not all of the long-term-reconstituting activity of the population. Fleming et al., *J. Cell. Biol.*, 122:897-902 (1993) have compared the reconstitution potentials of Hoechst 33342-sorted G<sub>0</sub>-G<sub>1</sub> and S-G<sub>2</sub>M fractions of Thy1.1<sup>hi</sup>Lin<sup>lo</sup> Sca-1<sup>+</sup> stem cells. They observed 18% of the cells in S-G<sub>2</sub>M, and reported a lower reconstitution capacity of this subset of cells. However, as shown here, Hoechst fluorescence

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in HSC reflects mdr-like efflux activity, not DNA content, unless the efflux activity is blocked with inhibitors such as verapamil. Therefore, the Hoechst content of their HSC would be lower than other cells in their population, and  
5 even cycling HSC would fall into their G<sub>0</sub>-G<sub>1</sub> peak (and perhaps below it). Therefore, the reconstitution potential of the cycling fraction was probably underestimated. They also examine the cell cycle profile of the Rh123<sup>low</sup> sub-set of Thy1.1<sup>b</sup> Lin-/<sup>b</sup>Sca-1+ cells by propidium iodide staining,  
10 and find approximately 3% of these cells are in S-G<sub>2</sub>M. This figure correlates well with the number of our SP cells in S-G<sub>2</sub>M, and it is expected that these populations are qualitatively extremely similar.

The ability to obtain populations of cycling stem  
15 cells also has important implications for hematopoietic stem cell therapy, including administration of the cells, as obtained by the present method as modified, such as by the introduction of an exogenous gene encoding a protein of interest (e.g., a therapeutic protein). Previous attempts  
20 to infect unmanipulated murine and human HSCs with recombinant retroviruses have met with mixed success, presumably at least in part due to the inability of retroviral vectors to integrate in quiescent cells. As shown here, only 1-3% of HSC are in cycle. The  
25 purification of the rare population of stem cells that are naturally replicating *in vivo* provides an alternative to transduction strategies based on attempts to influence the cell cycle status of stem cells.

The invention is further illustrated in the following  
30 examples.

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EXEMPLIFICATIONExample 1: Characterization of Hoechst 33342 fluorescence on whole murine bone marrow.Preparation of Hoechst 33342-stained Murine Bone Marrow

5 Murine bone marrow was extracted from the femurs and tibias of C57B1/6 mice, a single cell suspension was made by passage of the bone marrow through an 18 gauge needle, and the cells were pelleted by centrifugation. The bone marrow cells were resuspended at  $10^6$  cells per ml in pre-  
10 warmed DMEM contain 2% fetal calf serum, 1mM HEPES, 50 units/ml Penicillin, 50  $\mu$ g/ml Streptomycin, and 5 $\mu$ g per ml Hoechst 33342 (Sigma) and incubated for 90 minutes at 37°C. The resolution of these populations is sensitive to the staining time and the Hoechst dye concentration (Elwart,  
15 J.W. and Dormer, P., *Cytometry*, 11:239-43 (1990)).

After Hoechst staining, cells are maintained at 4°C until FACS analysis. Any antibody staining or manipulation is performed at 4°C following the Hoechst stain. After the final manipulation, the bone marrow cells were resuspended  
20 in HBSS+ containing 2 $\mu$ g/ml propidium iodide (PI). The addition of PI did not affect the Hoechst staining profile, but allowed exclusion of dead cells as described below.

Flow Cytometer Set-Up

Analysis and sorting were performed on a dual laser  
25 FACStar-plus or Facsvantage (Becton Dickenson). The Hoechst dye was excited by the first Argon laser at 350nm and its fluorescence was measured at two wavelengths using a 450 band pass (BP) 20 and a 675 edge filter long pass (EFLP) optical filter (Omega Optical, Brattleboro VT). A  
30 610 dichroic mirror short pass (DMSP) was used to separate the emission wavelengths. A 640 EFLP with 640 DMLP have

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also been used with an Enterprise laser (Coherent). Propidium iodide (PI) fluorescence was also measured through the 675 EFLP (having been excited at 350 nm). Hoechst "blue" represents the 450 BP filter, the standard analysis wavelength for Hoechst 33342 DNA content analysis. Cells positive for PI were seen on the far right of the Hoechst "red" (675 EFLP) axis shown, and excluded. Both Hoechst blue and red fluorescence are shown on a linear scale. Optimal cvs off of the first laser are necessary to finely resolve the stem cell population. The second Argon laser at 488 nm was used to excite standard fluorochromes (e.g., fluorescein or phycoerythrin if necessary). No cross compensation was necessary. The gating on forward and side scatter was not stringent, only erythrocytes and debris were excluded. Re-analysis of sorted populations showed purity greater than 98%.

Flow Cytometry Profile: Identification of the Side Population (SP) Cells

The Hoechst 33342-stained murine bone marrow is placed on the flow cytometer. Since the Hoechst fluorescence is analyzed on a linear scale, optimal cvs are obtained with a relatively low sample differential, but if the cells are resuspended at a sufficiently high concentration, they may still be run at 3000-5000 cells per second. The sample can be maintained at 4°C. Initially, the voltages of Hoechst-detectors are set so that the bulk identifiable population is centered when Hoechst-Blue is displayed on the Y axis, and Hoechst-red is displayed on the X axis. Red blood cells show up in the far lower left corner as cells which indicate little or no Hoechst fluorescence. With the UV laser in the first position, the red blood cells can be thresholded out. On an Enterprise laser, the UV beam is in the second position, so this is not possible. Dead cells fluoresce with propidium iodide, and show up on the far



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right of the profile, as very positive on the red axis. A live gate is drawn to include only live nucleated cells on the basis of the above parameters. In order to identify the region containing the stem cells, a sufficient number of data points (i.e., events) must be collected during flow cytometry analysis (100,000 events, where 10,000 events is standard). This allows the small side-population (SP) to be readily identified, as shown boxed in Figure 1A. When this region is purified by fluorescence activated cell sorting (FACS) and when used in transplantation assays, described below, this region contains all of the stem cell activity in C57B1/6 mice. The frequency of cells in this region is close to 0.1%.

15 Cell Surface Characterization of the Hoechst "Side Population"

Antibody staining was performed as follows: Hoechst-stained bone marrow was suspended in Hanks Balanced Salt Solution (HBSS) containing 2% fetal calf serum, 1mM HEPES, penicillin, and streptomycin (HBSS+) at  $10^8$  cells per ml. The antibodies that make up the lineage cocktail were added at 1/50 to 1/100 dilutions (after being titered for this cell concentration). The cocktail is comprised of the following: CD4 (GK1.5, Becton Dickenson); CD8 (53-6.7, Becton Dickenson); CD5 (53-7.3, Pharmingen); B220 (RA3-6B2, Caltag); Mac-1 (M1/70.15, Caltag); Gr-1 (RB6-8C5, Pharmingen). The mixture was incubated on ice for 10 minutes, then the bone marrow was washed once in excess HBSS+ and the cells were pelleted through a serum cushion. All washes were performed in this manner. The cells were resuspended in media containing Goat anti-rat antibody conjugated to phycoerythrin (mouse-serum adsorbed, Caltag), and incubated for 10 minutes on ice. After washing, the cells were resuspended in 1/3 volume rat serum (Cappel) and

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2/3 HBSS+. After 10 minutes on ice, biotinylated Sca-1 antibody (E13 161-7) was added for 10 minutes on ice. After washing, the cells were stained with avidin-FITC (Becton Dickenson) for 10 minutes on ice. Alternatively, 5 Goat-anti-rat-FITC may be used to detect the lineage antibodies, and streptavidin-PE for Sca-1.

After the final wash, the bone marrow cells were resuspended in HBSS+ containing 2 $\mu$ g/ml propidium iodide (PI). The addition of PI did not affect the Hoechst 10 staining profile as shown in Fig. 1A, but allowed exclusion of dead cells as described above. In sorting experiments, the bone marrow was sometimes magnetically pre-enriched for Sca-1 positive cells using the MACS (Miltenyi Biotec) and streptavidin microbeads. This resulted in a 5 to 10 fold 15 enrichment, and did not affect enrichment data. Cells were sorted into glass tubes containing 100% fetal calf serum. An aliquot was removed and reanalyzed to establish high purity, and cells were washed and counted prior to dilution for bone marrow transplantation.

20 The Sca/lin profile on whole bone marrow is shown in Figure 1B with the region shown by us and others to contain all of the stem cell activity in the mouse indicated. This region contains between 1-5% of the cells in the bone marrow after live gating out the red blood cells and dead 25 cells as described above. This number varies depending on which secondary conjugates are used to detect Sca-1 or the lineage cocktail.

If the region indicated in Fig. 1B is used as a live gate (in conjunction with the gate to exclude red and dead 30 cells), the Hoechst profile is heterogeneous as shown in Fig. 1C. 5-10% of the cells fall into the side population. If the region indicated in Figure 1A is used as the SOLE LIVE GATE on the whole population of murine bone marrow cells, the sca/lin profile of those cells is shown in 35 Figure 1D. In the example shown, approximately 75% of the

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cells fall into the Sca-1<sup>positive</sup> lineage<sup>negative</sup> region shown to contain all of the stem cells. Reducing the size of the side population box to avoid contamination from the bulk population increases the apparent cell surface homogeneity of the SP cells up to about 95%.

This procedure to characterize the cell surface expression of the Sca-1 and lineage antigens can be repeated for any marker alone or in combination. The following characterizations have thus been made for the human SP cells: glycophorin A<sup>neg</sup>, CD38<sup>low/neg</sup>, CD13<sup>neg</sup>, CD15<sup>neg</sup>, CD19<sup>neg</sup>, CD20<sup>neg</sup>, and CD34<sup>neg</sup>. The following characterizations have been made for the murine SP cells: c-kit<sup>pos</sup>, Sca-1<sup>pos</sup>, Gr-1<sup>neg/low</sup>, Mac-1<sup>neg/low</sup>, CD4<sup>neg/low</sup>, CD8<sup>neg</sup>, B220<sup>neg</sup>, CD5<sup>neg</sup>, CD43<sup>pos</sup>, CD45<sup>pos</sup>, M169<sup>pos</sup>, AA4<sup>neg/low</sup>, class IMHC<sup>pos</sup>, class II MHC<sup>neg</sup>, Rhodamine 123<sup>low</sup> and Wheat germ agglutinin (WGA)<sup>pos</sup>

Example 2: Long Term Bone Marrow Transplants Using Hoechst-purified stem cells

Competitive Repopulation Assay Using Hoechst-purified stem cells

To examine the functional properties of the cells purified in Example 1, long term bone marrow transplantation experiments were performed. For the competitive repopulation assay congenic C57B1/6 mouse strains which differ at the Ly-5 locus were used (Scheid, M.P. and Triglia, D., *Immunogenetics*, 9:423-433 (1979)). The Ly-5 antigen is found on the surface of all nucleated peripheral blood cells and the two allelic variants (i.e., Ly-5.1 and Ly-5.2) are readily distinguished by specific monoclonal antibodies. Peripheral blood was collected from transplant recipients at multiple time points and assayed by flow cytometry for the proportion of the two Ly-5 alleles present in each blood lineage.

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Typically, bone marrow was extracted from femurs and tibias of 10 male C57B1/6-Ly-5.1 mice (National Cancer Institute) 6-10 weeks in age and purified as described in Example 1. Stem cells were purified on the basis of the combination of the Hoechst side population and Sca-1+lin<sup>low</sup> fluorescence, as described in Example 1. This population was selected as follows: first, a live gate was defined using Hoechst red and blue axis to exclude dead cells and debris. After collecting 10<sup>5</sup> events within this live gate, the SP population is able to be clearly defined. A new live gate is established on this population, as shown boxed in Figure 1A, and the fluorescence of Sca-1 and lineage markers on SP cells was displayed. This allows a clear definition of Sca-1+ Lin<sup>low</sup> cells, as the bulk of the SP cells stand out immediately as falling into the region defined in Figure 1B. A sorting gate is then defined as cells which fall into both the region boxed in Figure 1A and in Figure 1B. Cells are purified by FACs and when they are reanalyzed, the purity is greater than 98%.

These purified cells were counted and mixed with unfractionated bone marrow cells (i.e., competitor cells) obtained from two male C57B1/6-Ly-5.2 mice (Jackson Laboratory), 6-10 weeks in age, and the mixtures were introduced into female C57B1/6-Ly-5.2 recipient mice (i.e., recipients) (Jackson Laboratory). The recipients were 6 to 12 weeks in age, and maintained on acidified water. The recipients were irradiated with 1100 rads given in two doses (620 rads and 480 rads) at least 2 hours apart. Transplanted cells were given intravenously by retro-orbital injection under methoxyflurane (Pittman Moore) anesthetic. Peripheral blood was taken by retro-orbital puncture under methoxyflurane anesthesia 4 months post-transplant. All animal care was in accordance with institutional guidelines. Red blood cells were removed and

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the nucleated peripheral blood cells were stained with biotinylated anti-Ly-5.1 antibody which was detected with streptavidin-PE (Molecular Probes). Sometimes, the blood samples were subdivided and co-stained with directly  
5 conjugated lineage specific antibodies against Thy-1, B220, Gr-1, and/or Mac-1 (Pharmingen). These blood samples were analyzed on the Facstar-plus, or a Facscan (Becton Dickinson). The anti-Ly-5.2 and anti-Ly-5.1 hybridomas used were 104.2.1 and A20.1.7 respectively (gifts from D.  
10 Pardoll). Other anti-Ly-5.2 and anti-Ly-5.1 hybridomers are available for use (Spangrude, G.J., et al., *Science*, 241:58-62 (1988)).

The results are shown in Table 1. The first column displays the number of Hoechst-purified stem cells (HSC).  
15 The second column displays the number of competitor cells introduced (see below). N represents the number of transplant recipients at the time of analysis (usually the same as the number initially transplanted) in each group. The mean is the mean number of HSC-derived (Ly-5.1+)  
20 nucleated peripheral blood cells present in recipients 4 months post-transplant, and SD is the standard deviation. Enrichment is calculated as the mean percentage contribution Ly-5.1+ cells in the peripheral blood per  
purified cell introduced, divided by the mean percentage  
25 contribution of Ly-5.2+ cells in the peripheral blood per unfractionated Ly-5.2 cell introduced. All numbers are rounded to two significant digits.

As shown in Table 1, the peripheral blood was analyzed 4 months post-transplant, ensuring that most of the cells  
30 present were derived from HSC. Furthermore, the contribution of the SP cells to the total stem cell activity was maintained at the same level for at least 12 months post transplant. In addition, the SP cells have been shown to contribute to all major blood lineages.  
35 Interestingly, as seen in Table 1, lower does of purified

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SP cells routinely appear to give rise to apparently higher enrichments, suggesting that there is some complex regulation when large numbers of purified stem cells are introduced into recipients.

Table 1. Competitive Repopulation by Hoechst-Purified Stem Cells

HSC	compet $\times 10^3$	n	mean	SD	enrichment
1000	200	5	79	11	720
300	200	4	54	14	780
100	200	4	43	3	1500
1000	400	4	52	2	400
300	400	3	26	9	470
100	400	5	29	15	1600

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5 Competitive repopulation assay using various regions of the hematopoietic stem cell populations in Figure 1E

Each of the three regions in Figure 1E was purified as described in Example 1 and tested for stem cell activity using the competitive repopulation assay described above.

10 Bone marrow from C57B1/9-Ly-5.1 mice was magnetically enriched for Sca-1 antibody staining cells to afford approximately 8-fold enrichment for stem cell activity. Sca-1 is known to mark all of the stem cells in this strain of mice.

15 The results are shown in Table 2. The first column indicates the region of cells tested. The second column displays the number of purified cells transplanted. The third column displays the number of competitor cells introduced. N represents the number of transplant  
20 recipients at the time of analysis (usually the same as the number initially transplanted) in each group. The mean is the mean number of nucleated peripheral blood cells derived from the fractionated bone marrow present in recipients 4 months post-transplant, and SD is the standard deviation.  
25 Enrichment is calculated as the mean % Ly-5.1<sup>+</sup> cells in the peripheral blood per purified cell introduced, divided by the mean percentage contribution of Ly-5.2<sup>+</sup> cells per unfractionated Ly-5.2 cell introduced. All numbers are rounded to two significant digits.



Table 2. Competitive Repopulation Assay using Three Regions of the SP Cells.

Fraction	# cells x 10 <sup>-3</sup>	#5.2 x 10 <sup>-3</sup>	n	% 5.1 <sup>+</sup> PBL	SD	Enrichment
SP	2	1000	6	56	5	640
SP	1	1000	7	41	5	698
SP	0.5	1000	7	30	8	960
upper SP	8.2	500	5	31	11	28
NOT SP	20	500	5	8	3	2

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Radioprotective ability of purified stem cells

Having established average enrichments of HSC activity of approximately 1000 fold with the SP cells in the competitive repopulation assays, the ability of the SP  
5 cells to protect recipients from lethal irradiation was investigated.

Purified HSC (male C57B1/6-Ly-5.1 derived, Hoechst SP, Sca-1+, lin<sup>low</sup>) were introduced into female C57B1/6-Ly-5.2 recipients as described above for the competitive  
10 repopulation assay. Results from three independent experiments are shown in Table 3. The number of purified HSCs introduced into the recipient mice are shown in the first column. The number of animals transplanted in each group is shown in the second column. The percentage of  
15 animals surviving at least 4 months post-transplantation is shown in the third column. The mean percent Ly-5.1 nucleated peripheral blood cells in the transplant recipients 4 months post-transplant is shown in column four. The standard deviation (SD) is shown in column five.  
20 All numbers are rounded to two significant digits.

As shown by data from three separate experiments in Table 3, approximately 150 purified cells radioprotected 50% of recipients. The mean contribution of the SP cells to hematopoiesis in the peripheral blood of survivors was  
25 at least 81%. Although others have observed a higher level of radioprotective activity from their purified populations, the mean contribution of the transplanted stem cells to hematopoiesis in their survivors was considerably lower (Spangrude, G.J., et al., *Science*, 241:58-62 (1988);  
30 Fleming, W.H., et al., *J. Cell Biol.*, 122:897-902 (1993); Li, C.L., Johnson, G.R., *Exp. Hematol.*, 20:1309-15 (1992); Spangrude, G.J. and Scollay, R., *Exp. Hematol.*, 18:920-926 (1990)). This may reflect qualitative differences in the purified populations, or possibly differences in animal

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husbandry practices which affect survival after lethal irradiation. In addition, the 12-day spleen colony formation (CFU-s) of this population was measured to be approximately 1 CFU-s(12) per 35 purified cells injected.

- 5 This frequency correlates well with the CFU-s(12) forming frequency of the Rhodamine-123<sup>low</sup> sub-population of Sca-1<sup>+</sup> lin<sup>low</sup> Thy-1.1<sup>low</sup> cells (Spangrude, G.J. and Johnson, G.R., *Proc. Natl. Acad. Sci. U.S.A.*, 87:7433-7 (1990)).

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Table 3. Radioprotective Ability of Purified Stem Cells

HSC	n	% survival	mean	SD
25	10	0	0	-
50	10	10	12	-
100	20	25	81	11
200	10	80	87	10
500	10	70	89	6
25	20	4	1	-
50	19	0	0	-
100	29	24	83	18
200	20	60	89	6
0	15	0	0	-
100	20	40	85	16
250	20	45	95	4
500	20	70	97	1
1000	19	68	97	1

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Example 3: Inhibition of Multiple Drug Resistant protein with Verapamil

Since it is known that several vital dyes are actively pumped out of the cells by the multidrug resistance protein (mdr, or p-glycoprotein), the possibility that the exceptionally low Hoechst fluorescence was due to higher mdr activity in murine hematopoietic stem cells was investigated (Chaudhary, P.M. and Roninson, L.B., *Cell*, 66:85-94 (1991)).

Whole murine bone marrow was stained for 90 minutes in 5µg/ml Hoechst 33342 as described in Example 1 with 50 µM verapamil or without verapamil (Sigma). After staining, samples were kept on ice until flow cytometry analysis as described in Example 1.

The results, which are shown in Figures 2A (without verapamil) and 2B (with verapamil), demonstrate that purified stem cell population cannot be isolated in the presence of verapamil using the method of the present invention. Therefore, it is reasonable to expect that the stem cells are uniquely low in Hoechst fluorescence due to mdr or an mdr-like mediated efflux of the dye. This very high dye efflux activity may be due to a higher level of p-glycoprotein on the surface of HSC, a higher level of activity of the p-glycoprotein present, or an mdr-like activity not yet identified. Antibody studies on the expression of the product of the MDR1 gene in human hematopoietic cells suggest that p-glycoprotein is in fact expressed quite widely, and that up to 65% of bone marrow cells may express mdr (Chaudhary, P.M. and Roninson, L.B., *Cell*, 66:85-94 (1991); Drach, D., et al., *Blood*, 80:2729-34 (1991)). In light of this, a purification strategy based on functional properties, such as the one described herein, will likely be more powerful than a scheme based on the level of mdr cell surface expression (e.g., antibody-based).

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Example 4: Analysis of the Cell Cycle Status of Purified Hematopoietic Stem Cells

Initial purification of the HSC was as described in Example 1. After sorting to 98% purity, several thousand  
5 sorted stem cells were incubated for 60 minutes at 37°C in 10 ml DMEM containing 2% fetal calf serum, 1mM HEPES, Penicillin, Streptomycin, 10 µg per ml Hoechst 33342, and 50 µM verapamil. Flow cytometry analysis was as described in Example 1. Propidium iodide stain: Stem cells purified  
10 as described above were pelleted by centrifugation and resuspended in 0.1% NaCitrate, 50 µg/ml propidium iodide. After incubation on ice for 10 minutes, the cells were analyzed by standard flow cytometry procedures using 488 nm excitation. The results are shown in Fig. 3A and Fig. 3B.

15 An aliquot of the SP cells was removed and transplanted as the "1x sorted" group in column 1. The rest of the sorted cells were incubated for 60 minutes in DMEM as described in Figure 1, containing 10 µg/ml Hoechst 33342 and 50 µM Verapamil. These were resuspended in HBSS  
20 including 2 µg/ml of propidium iodide, and sorted on the flow cytometer as described in Figure 1. Several thousand cells were first allowed to pass through the laser without subfractionation. These were transplanted as the "2x sorted" group. Large differences in the reconstitution  
25 potential between the 1x sorted and 2x sorted groups could have indicated damage of the HSC by the sorting conditions. The remaining cells were sorted into either 2n ( $G_0$ - $G_1$ ) or >2n ( $S$ - $G_2$ M) groups, and transplanted as indicated.

When cell-cycle analysis was performed in this way,  
30 the number of cells in  $S$ - $G_2$ M (i.e., cycling HSCs) was found to range between 1 and 3% of the purified cells (Fig. 3A). This figure correlates well with the number shown to be in  $S$ - $G_2$ M by propidium iodide staining of the purified cells (Fig. 3B). This is much lower than the fraction of cells

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in S-G<sub>2</sub>M in whole bone marrow (20%), and in other populations enriched for stem cells (Fleming, W.H., et al., *J. Cell Biol.*, 122:897-902 (1993)). This low number of cells in cycle supports the widely held view that HSC from  
5 normal bone marrow are largely quiescent.

With a view to using cycling stem cells as targets for retroviral-mediated gene transfer, the relative engraftment potential of stem cells that were in G<sub>0</sub>-G<sub>1</sub> (i.e., quiescent HSCs) versus S-G<sub>2</sub>M was compared. These subsets of the SP  
10 population were purified by flow cytometry using the verapamil-blocking strategy for cell cycle analysis described in Example 1, and tested in the competitive repopulation assay described in Example 2. Table 4 shows the results from the experiment.

15 One experiment is shown with the analysis performed at both 2 months and 11 months post-transplant. The number of purified or sub-fractionated HSC transplanted per mouse is shown in the second column (HSC). The number of unfractionated Ly-5.2 competitor bone marrow cells co-  
20 transplanted is indicated in the third column, and the number of mice at the time of analysis (n) in the fourth column. The mean shown is the mean percentage of Ly-5.1+ nucleated peripheral blood cells at the time post-transplant indicated, and SD is the standard deviation.  
25 The enrichment is calculated as in Table 1. All numbers are rounded to two significant digits.

These data demonstrate several points. Firstly, the contribution of the G<sub>0</sub>-G<sub>1</sub> HSC to the peripheral blood is almost identical to that of the S-G<sub>2</sub>M HSC, and both of  
30 these populations are virtually identical in activity to HSC which have not been sub-fractionated (1x and 2xx sorted HSC). Secondly, a passage of HSCs through the flow cytometer twice (2x sorted; exciting the fluorescence of a DNA-binding dye with a UV laser) does not seem to affect

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the capacity of the HSC to repopulate the bone marrow.  
Thirdly, the percentage of peripheral blood cells derived  
from the sorted cells is almost identical at 2 months and  
11 months, reflecting the very long term stem cell activity  
5 of all of these populations.



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Table 4. Competitive Repopulation With Cycling and Non-Cycling Cells

	Group	HSC	compet $\times 10^3$	n	mean	SD	enrichment
2 Month	1x sorted	400	400	9	44	10	800
	2x sorted	400	400	7	56	6	1300
	2x sorted	200	200	6	52	16	1100
	G <sub>0</sub> -G <sub>1</sub>	400	400	6	47	9	870
	G <sub>0</sub> -G <sub>1</sub>	200	200	7	62	17	1600
	S-G <sub>2</sub> M	200	200	5	64	2	1800
11 Month	1x sorted	400	400	7	51	14	1000
	2x sorted	400	400	6	60	8	1500
	2x sorted	200	200	6	56	26	1300
	G <sub>0</sub> -G <sub>1</sub>	400	400	6	54	7	1200
	G <sub>0</sub> -G <sub>1</sub>	200	200	7	60	11	1500
	S-G <sub>2</sub> M	200	200	5	63	20	1700

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5 Example 5: Characterization of Hoechst 33342 Fluorescence  
on Whole Human Bone Marrow and Human Cord Blood

Cell surface characterization of human SP cells

Hematopoietic stem cells from whole human bone marrow  
10 and human umbilical cord blood were identified as described  
in Example 1, except as described below. Human SP cells  
were characterized following the procedure for cell surface  
characterization of the mouse SP cells described in Example  
1. In some samples, there are some CD34-positive cells at  
15 the TOP of the SP region, near the bulk population of  
cells. But the majority of cells in the SP region in  
several samples of human marrow that we have examined are  
characterized as follows: Glycophorin A<sup>neg</sup>, CD38<sup>low</sup>, CD13<sup>neg</sup>,  
CD15<sup>neg</sup>, CD19<sup>neg</sup>, CD20<sup>neg</sup> and CD34<sup>neg</sup>. The human cells run at a  
20 lower frequency, closer to 0.03-0.05% in the bone marrow.  
Typically, this bone marrow has been depleted of red blood  
cells and platelets, by a ficoll density centrifugation.  
The results are shown in Figures 4A and 4B.

25 Example 6: Characterization of Hoechst 33342 Fluorescence  
on Porcine Bone Marrow and Monkey Bone Marrow

Hematopoietic stem cells from porcine bone marrow and  
monkey bone marrow were identified as described in  
Example 1, except as indicated below. Porcine bone marrow  
30 and Rhesus macaque monkey bone marrow were characterized  
following the procedure for cell surface characterization  
of the mouse SP cells described in Example 1. In addition,  
fresh bone marrow was depleted of red blood cells in the  
case of Rhesus bone marrow by a ficoll density gradient.  
35 Nucleated cells were counted and resuspended at  $10^6$   
nucleated cells per ml in pre-warmed DMEM/10% FCS, 1mM  
HEPES, and 50 units per ml penicillin and 50  $\mu$ g per ml  
streptomycin. Hoechst 33342 dye was added to a

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concentration of 5  $\mu\text{g/ml}$ , and the cells were incubated at 37°C for 120 minutes. Bone marrow samples were run on the flow cytometer using the set-up described for the murine bone marrow.

- 5 The results are shown in Figures 5A and 5B. The arrows indicate the presence of cells in the same region murine stem cell activity is observed (see Figure 1A). The region represents 0.05% to 0.1% of the nucleated cell population in both of these animal models. The phenotype of the Rhesus monkey SP cells are as follows: CD34<sup>neg</sup>,  
 10 CD38<sup>low/neg</sup>, CD4<sup>neg</sup>, CD8<sup>neg</sup>, Glycophorin A negative, CD61<sup>neg</sup>, and CD66<sup>neg</sup>.

Example 7: Rhesus Long Term Culture Initiating Cell  
 15 (LTCIC) Limiting Dilution Experiments

- The LTCIC assay is considered by many in the field to represent the best *in vitro* assay for HSCs. As described below, this assay was performed on hematopoietic stem cells from Rhesus monkeys. Hematopoietic stem cells from Rhesus  
 20 monkeys were identified as described in Example 1. The LTCIC assays were performed as described in Sutherland, H.J., et al., *Proc. Natl. Acad. Sci.*, 87:3584-3588 (1990). The results are shown in Table 5.

Table 5. Rhesus LTCIC Limiting Dilution Experiments.

Unfractionated	Unfract./HO-stained	CD34 <sup>pos</sup> /CD38 <sup>neg</sup>	SP <sup>+</sup> /CD34 <sup>neg</sup>
1/4 x 10 <sup>3</sup>	1/5 x 10 <sup>3</sup>	1/55	1/6
1/12 x 10 <sup>3</sup>	1/15 x 10 <sup>3</sup>	1/53	1/8
1/12 x 10 <sup>3</sup>	1/8 x 10 <sup>3</sup>	1/51	1/7

Numbers given represent number of LTCIC forming units per cell plated in a limiting dilution-type assay. Bone marrow from three separate monkeys is shown. As shown in

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Table 5, the hematopoietic stem cell activity on a per cell  
5 basis of the SP cells that are CD34 negative (column 4) is  
significantly higher than the CD34<sup>pos</sup>/CD34<sup>neg</sup> cells  
(column 3), which are considered in the art to define stem  
cells, from the same animal. The controls are  
unfractionated (column 1) and unfractionated/Hoechst-  
10 stained (column 2) marrow. The unfractionated and CD34<sup>positive</sup>  
numbers of LTCIC are right in the range of LTCIC activity  
found in these populations in the literature. (Sutherland,  
H.J., et al., *Proc. Natl. Acad. Sci.*, 87:3584-3588 (1990)).  
Thus, the data indicates that in the monkey, SP cells,  
15 which are CD34<sup>neg</sup>, are highly enriched for LTICs.

#### EQUIVALENTS

Those skilled in the art will recognize, or be able to  
ascertain using no more than routine experimentation, many  
20 equivalents to the specific embodiments of the invention  
described specifically herein. Such equivalents are  
intended to be encompassed in the scope of the following  
claims.

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CLAIMS

We claim:

1. A method of purifying mammalian hematopoietic stem cells from a bone marrow cell sample comprising the steps of:
  - a) obtaining a mammalian bone marrow cell sample;
  - b) combining the cell sample of a) with a fluorescent, lipophilic vital dye which is a substrate for a multiple drug resistant protein, under conditions appropriate for cells in the cell sample to take up the dye;
  - c) exposing the combination produced in b) to an excitation wavelength which causes fluorescence of the dye;
  - d) assessing the fluorescence of the dye using an emission wavelength,wherein nucleated cells which contain the least amount of dye are purified mammalian hematopoietic stem cells.
2. A method of Claim 1 wherein the dye is Hoechst 33342 dye, the excitation wavelength is about 350 nm and the emission wavelength is from about 650 nm to about 675 nm.
3. A method of Claim 1 wherein the hematopoietic stem cells are human cells.
4. A method of Claim 3 wherein the hematopoietic stem cells are glycophorin A<sup>neg</sup>, CD38<sup>low/neg</sup>, CD13<sup>neg</sup>, CD15<sup>neg</sup>, CD19<sup>neg</sup>, CD20<sup>neg</sup>, and CD34<sup>neg</sup>.
5. A method of Claim 1 further comprising the step of combining the mammalian bone marrow sample with

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antibodies which bind cell surface markers of hematopoietic stem cells prior to step c).

- 5 6. Purified hematopoietic stem cells obtainable by the method of Claim 1.
7. A method of purifying mammalian hematopoietic stem cells from a bone marrow cell sample comprising the steps of:
  - 10 a) obtaining a mammalian bone marrow cell sample;
  - b) combining the cell sample of a) with a fluorescent, lipophilic vital dye which is a substrate for a multiple drug resistant protein, under conditions appropriate for cells in the cell sample to take up the dye;
  - 15 c) exposing the combination produced in b) to an excitation wavelength which causes fluorescence of the dye;
  - d) assessing the fluorescence of the dye using two different emission wavelengths simultaneously, wherein nucleated cells which contain the least amount of dye are purified mammalian hematopoietic stem cells.
  - 20
- 25 8. A method of Claim 7 wherein the dye is Hoescht 33342 dye.
9. A method of Claim 7 wherein the hematopoietic stem cells are human cells.
- 30 10. A method of Claim 9 wherein the hematopoietic stem cells are glycophorin A<sup>neg</sup>, CD38<sup>low/neg</sup>, CD13<sup>neg</sup>, CD15<sup>neg</sup>, CD19<sup>neg</sup>, CD20<sup>neg</sup>, and CD34<sup>neg</sup>.

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11. A method of Claim 7 wherein the excitation wavelength is about 350 nm and the two emission wavelengths are about 450 nm and about 675 nm.
- 5 12. A method of Claim 7 further comprising the step of combining the mammalian bone marrow cell sample with antibodies which bind cell surface markers of hematopoietic stem cells prior to step c).
- 10 13. Purified, mammalian hematopoietic stem cells obtainable by the method of Claim 7.
14. A method of purifying mammalian hematopoietic stem cells from a bone marrow cell sample comprising the steps of:
  - 15 a) obtaining a mammalian bone marrow sample;
  - b combining the cell sample of a) with Hoechst 33342 dye, under conditions appropriate for cells in the cell sample to take up the dye;
  - 20 c) exposing the combination in b) to an excitation wavelength of about 350 nm thereby causing fluorescence of the dye;
  - d) measuring the fluorescence of the dye using emission wavelengths of 450 nm and 675 nm simultaneously;
  - 25 e) determining the amount of dye uptake of each cell population of d) using flow cytometry, wherein nucleated cells which contain the least amount of dye are purified mammalian hematopoietic stem cells.
- 30 15. The method of Claim 14 wherein the hematopoietic stem cells are human cells.

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16. A method of Claim 15 wherein the hematopoietic stem cells are glycophorin A<sup>neg</sup>, CD38<sup>low/neg</sup>, CD13<sup>neg</sup>, CD15<sup>neg</sup>, CD19<sup>neg</sup>, CD20<sup>neg</sup>, and CD34<sup>neg</sup>.
- 5 17. A method of Claim 14 further comprising the step of combining the mammalian bone marrow cell sample with antibodies which bind cell surface markers of the hematopoietic stem cells prior to step c).
- 10 18. Purified, mammalian hematopoietic stem cells obtainable by the method of Claim 14.
- 15 19. A method of separating cycling hematopoietic stem cells from quiescent hematopoietic stem cells in a purified mammalian hematopoietic stem cell sample comprising the steps of:
- a) obtaining a purified mammalian hematopoietic stem cell sample;
  - 20 b) combining the cells of a) with a fluorescent, lipophilic, vital dye which is a substrate for a multiple drug resistant protein and binds DNA and an inhibitor of the multiple drug resistant protein under conditions appropriate for the cells to take up the dye and the inhibitor; and
  - 25 c) measuring the amount of dye present in the hematopoietic stem cells,
- wherein purified hematopoietic stem cells which take up the smallest amount of dye have a low concentration of DNA and are designated as quiescent hematopoietic stem cells, and purified hematopoietic stem cells
- 30 which take up a greater amount of dye have a greater amount of DNA and are designated cycling hematopoietic stem cells.



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20. A method of Claim 19 wherein the inhibitor of the multiple drug resistant protein is selected from the group consisting of: verapamil and anti-multiple drug resistant protein antibodies.
- 5
21. A method of Claim 19 wherein the hematopoietic stem cells are human.
22. A method of Claim 21 wherein the hematopoietic stem cells are glycophorin A<sup>neg</sup>, CD38<sup>low/neg</sup>, CD13<sup>neg</sup>, CD15<sup>neg</sup>, CD19<sup>neg</sup>, CD20<sup>neg</sup>, and CD34<sup>neg</sup>.
- 10
23. A method of Claim 19 wherein the purified hematopoietic stem cells are obtained using the method of purifying mammalian hematopoietic stem cells from a bone marrow cell sample comprising the steps of:
- 15
- a) obtaining a mammalian bone marrow cell sample;
  - b) combining the cell sample of a) with a fluorescent, lipophilic vital dye which is a substrate for a multiple drug resistant protein, under conditions appropriate for cells in the cell sample to take up the dye;
  - 20
  - c) exposing the combination produced in b) to an excitation wavelength which causes fluorescence of the dye;
  - 25
  - d) assessing the fluorescence of the dye using an emission wavelength,
- wherein nucleated cells which contain the least amount of dye are purified mammalian hematopoietic stem cells.
- 30
24. A method of Claim 19 wherein the purified hematopoietic stem cells are obtained using the method

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of purifying mammalian hematopoietic stem cells from a bone marrow cell sample comprising the steps of:

- a) obtaining a mammalian bone marrow cell sample;
- b) combining the cell sample of a) with a  
5 fluorescent, lipophilic vital dye which is a substrate for a multiple drug resistant protein, under conditions appropriate for cells in the cell sample to take up the dye;
- c) exposing the combination produced in b) to an  
10 excitation wavelength which causes fluorescence of the dye;
- d) assessing the fluorescence of the dye using two different emission wavelengths simultaneously, wherein nucleated cells which contain the least amount  
15 of dye are purified mammalian hematopoietic stem cells.

25. A method of separating cycling hematopoietic stem cells from quiescent hematopoietic stem cells in a  
20 purified, mammalian hematopoietic stem cell sample comprising the steps of:
  - a) obtaining a purified mammalian hematopoietic stem cell sample using the method of Claim 1;
  - b) combining the cells of a) with Hoechst 33342 dye  
25 and verapamil; and
  - c) measuring the amount of dye present in the hematopoietic stem cells using flow cytometry, wherein purified hematopoietic stem cells which take up the smallest amount of dye have a low concentration  
30 of DNA and are designated as quiescent hematopoietic stem cells, and purified hematopoietic stem cells which take up a greater amount of dye have a greater amount of DNA and are designated cycling hematopoietic stem cells.

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26. A method of Claim 25 wherein the hematopoietic stem cells are human.
27. A method of identifying an agent which is a  
5 fluorescent, vital dye which is lipophilic for use in purifying mammalian hematopoietic stem cells comprising the steps of:
- a) obtaining a mammalian bone marrow cell sample;
  - b) combining the cells of (a) with the agent under  
10 conditions appropriate for the cells to take up the agent;
  - c) exposing the combination of (b) to an excitation wavelength which results in fluorescence of the agent;
  - d) measuring the fluorescence at an emission  
15 wavelength,
- wherein if distinct populations of nucleated cells are observed in which one of the population of cells takes up the smallest amount of dye and the population is  
20 purified, mammalian hematopoietic stem cells, then the agent is a fluorescent, vital dye which is lipophilic for use in purifying hematopoietic stem cells.
28. A method of identifying an agent which is a  
25 fluorescent, vital dye which is lipophilic for use in purifying mammalian hematopoietic stem cells comprising the steps of:
- a) obtaining a mammalian bone marrow cell sample;
  - b) combining the cells of (a) with the agent under  
30 conditions appropriate for the cells to take up the agent;
  - c) exposing the combination of (b) to an excitation wavelength which results in fluorescence of the agent;

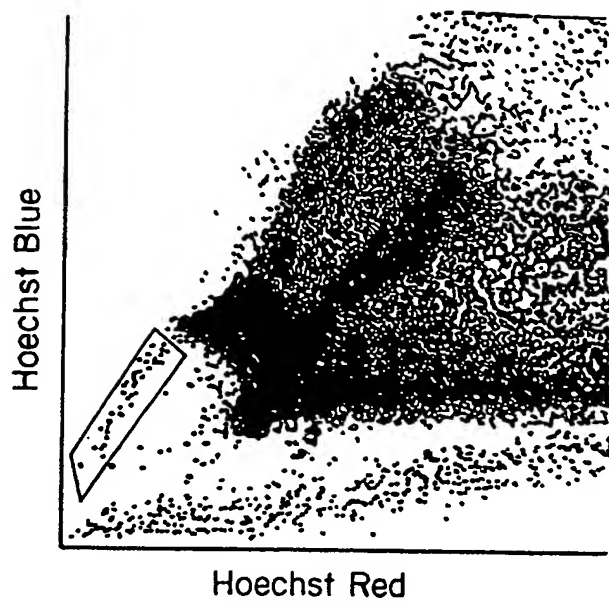
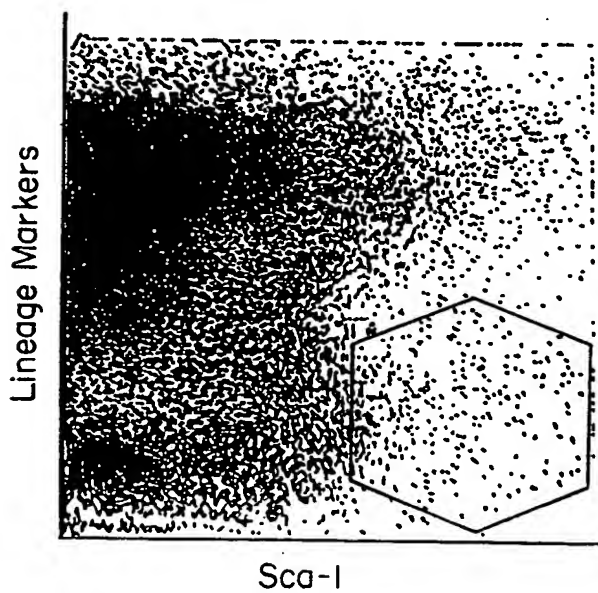
-50-

- d) measuring the fluorescence using two emission wavelengths simultaneously,  
wherein if distinct populations of nucleated cells are observed in which one of the population of cells takes up the smallest amount of dye and the population is purified, mammalian hematopoietic stem cells, then the agent is a fluorescent, vital dye which is lipophilic for use in purifying hematopoietic stem cells.
- 5
- 10 29. A method of transplanting bone marrow in a host comprising the step of introducing into the host the purified hematopoietic stem cells of Claim 1.
- 15 30. A method of providing a host with purified hematopoietic stem cells comprising the step of introducing into the host the hematopoietic stem cells of Claim 1.
- 20 31. A method of Claim 30 wherein the purified hematopoietic stem cells are cycling hematopoietic stem cells.
- 25 32. A method of in vivo administration of a protein comprising the step of transfecting a purified hematopoietic stem cell produced by the method of Claim 19 with a construct comprising DNA or RNA which expresses a protein of interest.
- 30 33. A method of Claim 32 wherein the purified hematopoietic stem cells are cycling hematopoietic stem cells.
- 35 34. A method of Claim 30 wherein the purified hematopoietic stem cells are quiescent hematopoietic stem cells.

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35. A method of Claim 32 wherein the purified hematopoietic stem cells are quiescent hematopoietic stem cells.
- 5 36. Purified hematopoietic stem cells that are CD34<sup>neg</sup>.
37. Purified hematopoietic stem cells of Claim 36 which are side population cells.
- 10 38. Purified hematopoietic stem cells of Claim 37 which exhibit at least one of the following characteristics: glycophorin A<sup>neg</sup>, CD38<sup>low/neg</sup>, CD13<sup>neg</sup>, CD15<sup>neg</sup>, CD19<sup>neg</sup> and CD20<sup>neg</sup>.
- 15 39. Purified hematopoietic stem cells of Claim 36 which exhibit at least one of the following characteristics: glycophorin A<sup>neg</sup>, CD38<sup>low/neg</sup>, CD13<sup>neg</sup>, CD15<sup>neg</sup>, CD19<sup>neg</sup> and CD20<sup>neg</sup>.

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**FIG. 1A****FIG. 1B**

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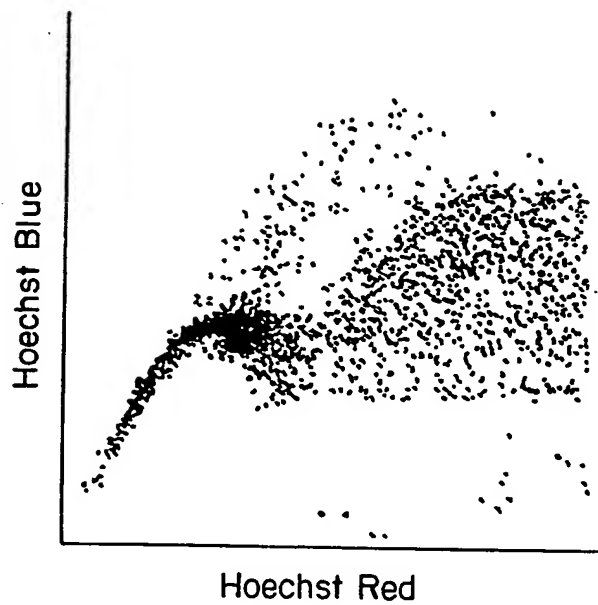


FIG. 1C

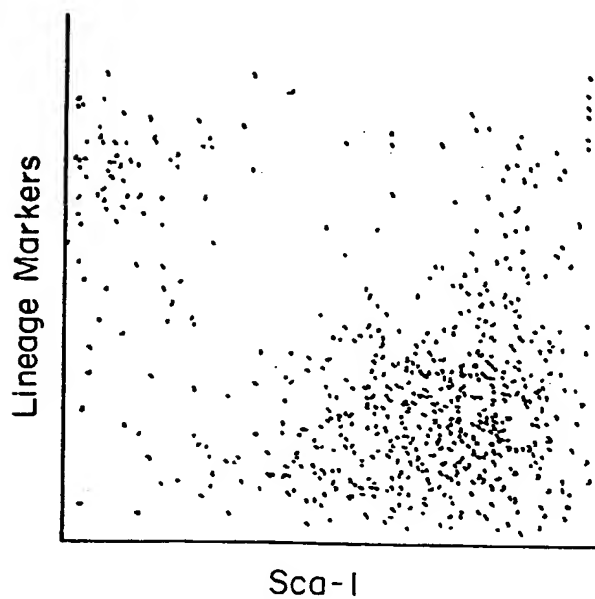
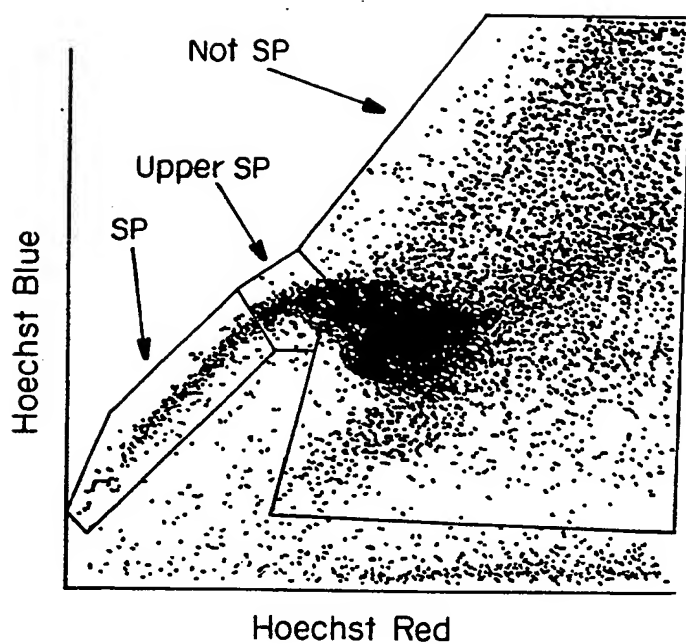


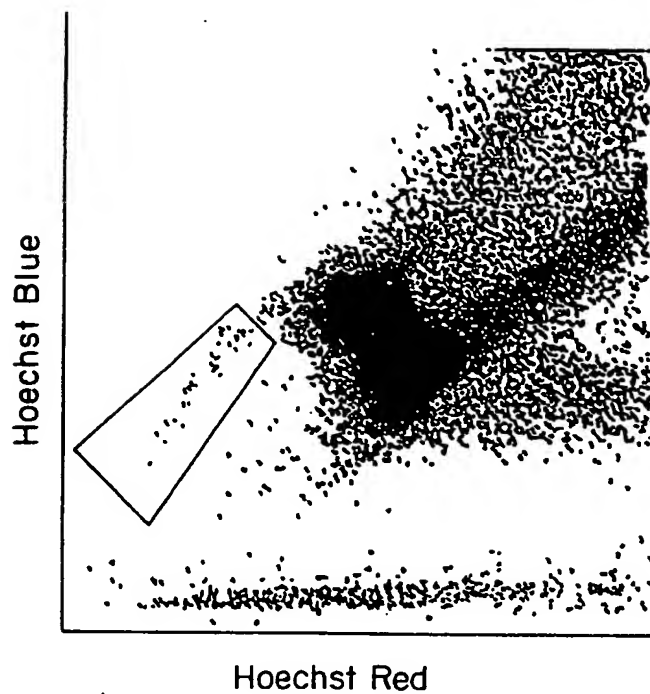
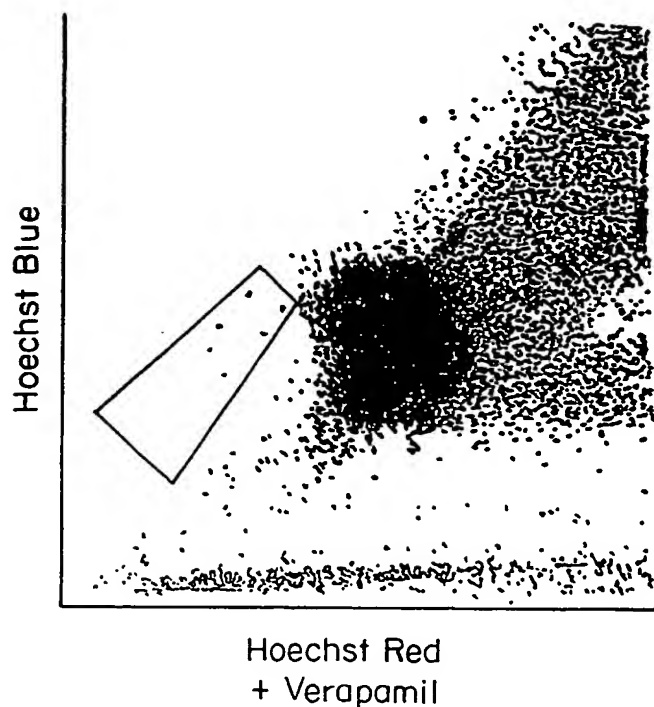
FIG. 1D

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**FIG. 1E**

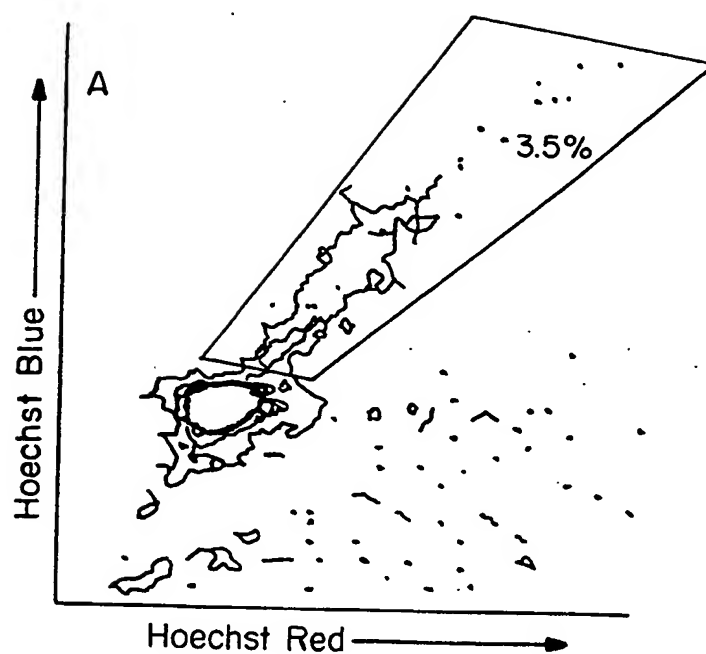
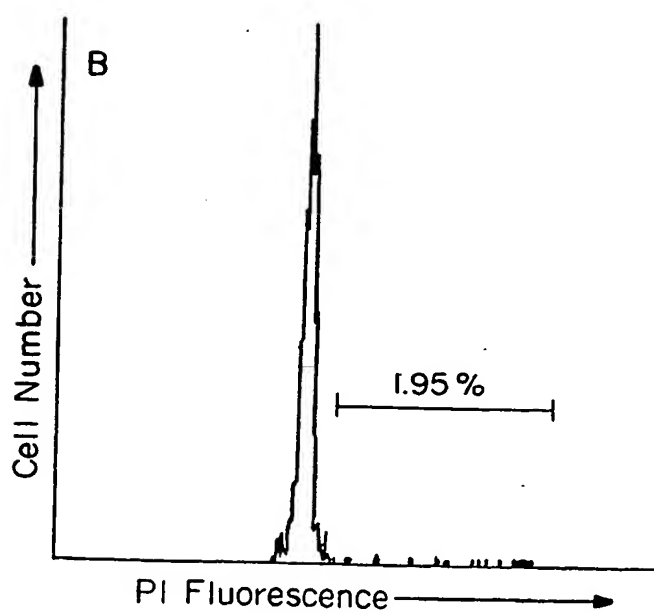


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**FIG. 2A****FIG. 2B**

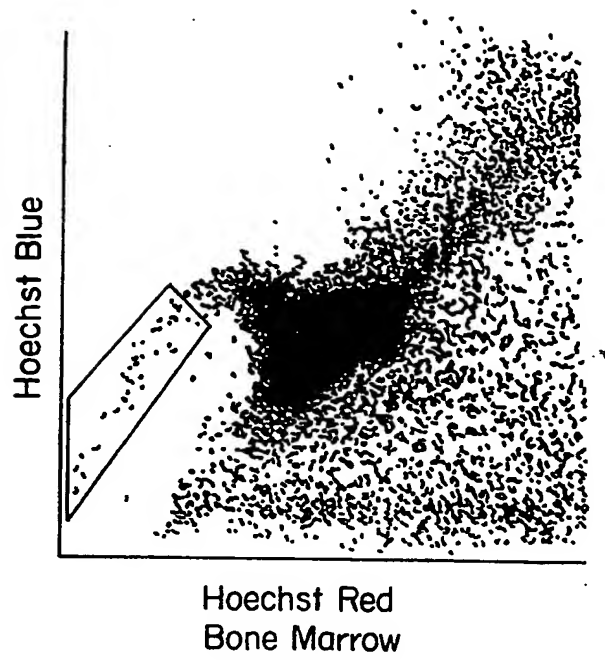
SUBSTITUTE SHEET (RULE 26)

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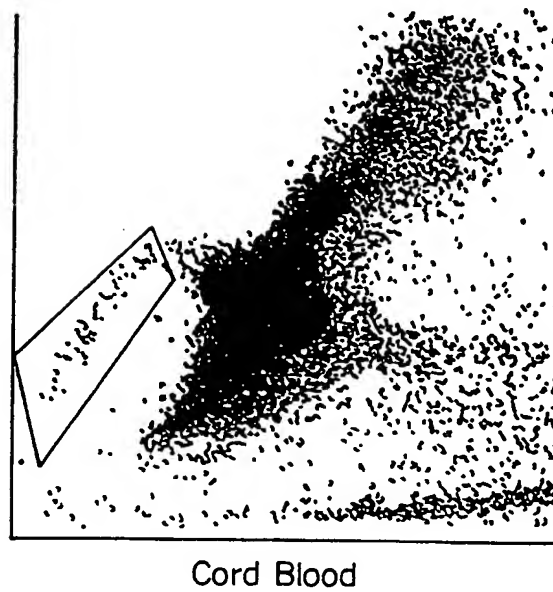
**FIG. 3A****FIG. 3B**

SUBSTITUTE SHEET (RULE 26)

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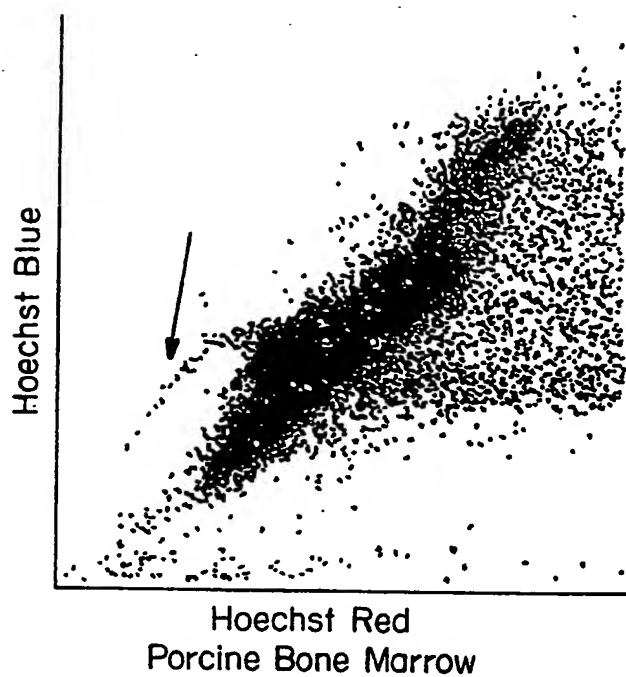


**FIG. 4A**

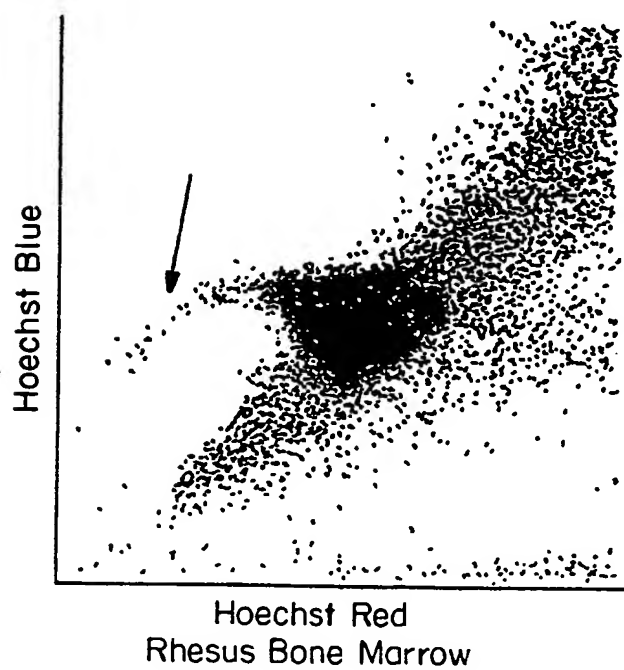


**FIG. 4B**

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**FIG. 5A**



**FIG. 5B**

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/09415

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N5/08 C12Q1/24 C12Q1/02 A61K35/28 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>THE JOURNAL OF CELL BIOLOGY, vol. 122, no. 4, August 1993, NEW YORK, N.Y., US, pages 897-902, XP000604719 W.H. FLEMING ET AL.: "FUNCTIONAL HETEROGENEITY IS ASSOCIATED WITH THE CELL CYCLE STATUS OF MURINE HEMATOPOIETIC STEM CELLS." cited in the application see page 897, abstract see page 901, left-hand column, line 29 - line 33</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1-39

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

27 September 1996

Date of mailing of the international search report

14. 10. 96

Name and mailing address of the ISA

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Authorized officer

Ryckebosch, A

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/Us 96/09415

## C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>EXPERIMENTAL HEMATOLOGY, vol. 21, no. 5, May 1993, LAWRENCE, KANS., US, pages 614-622, XP000604780 N.S. WOLF ET AL.: "IN VIVO AND IN VITRO CHARACTERIZATION OF LONG-TERM REPOPULATING PRIMITIVE HEMATOPOIETIC CELLS ISOLATED BY SEQUENTIAL HOECHST 33342-RHODAMINE 123 FACS SELECTION." see page 614, abstract see page 615, left-hand column, paragraph 6 - right-hand column, paragraph 2; figure 1 see page 621, left-hand column, paragraph 2</p>	1-39
A	<p>--- CELL, vol. 66, 12 July 1991, CAMBRIDGE, MA, US, pages 85-94, XP002014549 P.M. CHAUDHARY ET AL.: "EXPRESSION AND ACTIVITY OF P-GLYCOPROTEIN, A MULTIDRUG EFFLUX PUMP, IN HUMAN HEMATOPOIETIC STEM CELLS." cited in the application see page 85, right-hand column, paragraph 2 - page 86, left-hand column, paragraph 2</p>	1-39
A	<p>--- PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 87, October 1990, WASHINGTON US, pages 7433-7437, XP002014550 G.J. SPANGRUDE ET AL.: "RESTING AND ACTIVATED SUBSETS OF MOUSE MULTIPOTENT HEMATOPOIETIC STEM CELLS." cited in the application see page 7433, abstract</p>	1-39
A	<p>--- EXPERIMENTAL HEMATOLOGY, vol. 18, no. 3, March 1990, LAWRENCE, KANS., US, pages 248-256, XP000604657 J.W.M. VISSER ET AL.: "PURIFICATION OF PLURIPOTENT HEMATOPOIETIC STEM CELLS: PAST AND PRESENT." see page 250, left-hand column, paragraph 4 - right-hand column, paragraph 3</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1-39

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 96/09415

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 183, no. 4, 1 April 1996, NEW YORK, N.Y., US, pages 1797-1806, XP000604752 M.A. GOODELL ET AL.: "ISOLATION AND FUNCTIONAL PROPERTIES OF MURINE HEMATOPOIETIC STEM CELLS THAT ARE REPLICATING IN VIVO." see the whole document -----</p>	1-39

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/09415

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 29 - 35 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.☐ No protest accompanied the payment of additional search fees.